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Full Paper

Identification of novel potential acetate-oxidizing bacteria in an acetate-fed methanogenic chemostat based on DNA stable isotope probing

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Acetate is a significant intermediate of anaerobic fermentation. There are two pathways for converting acetate to CH_4 and CO_2: acetoclastic methanogenesis by acetoclastic methanogens, and syntrophic acetate oxidation by acetate-oxidizing bacteria (AOB) and hydrogenotrophic methanogens. Detailed investigations of syntrophic acetate-oxidizing bacteria (SAOB) should contribute to the elucidation of the microbial mechanisms of methanogenesis. In this study, we investigated the major phylogenetic groups of acetate-utilizing bacteria (AUB) in a mesophilic methanogenic chemostat fed with acetate as the sole carbon source by using DNA stable isotope probing (SIP) technology. The results indicated that acetoclastic methanogenesis and acetate oxidization/hydrogenotrophic methanogenesis coexisted in the mesophilic chemostat fed with acetate, operated at a dilution rate of 0.1 d^{-1}. OTU Ace13(9-17) (KU869530), Ace13(9-4) (KU667241), and Ace13(9-23) (KU667236), assigned to the phyla *Firmicutes* and *Bacteroidetes*, were probably potential SAOB in the chemostat, which needs further investigation. Species in the phyla *Proteobacteria*, *Deferribacteres*, *Acidobacteria*, *Spirochaetes* and *Actinobacteria* were probably capable of utilizing acetate for their growth. *Methanoculleus* was likely to be the preferred hydrogenotrophic methanogen for syntrophy with AOB in the chemostat.

Key Words: acetate-oxidizing bacteria; anaerobic digestion; DNA-SIP; methanogenesis; microbial community

Introduction

Anaerobic digestion is an environmentally friendly and effective technology for reducing organic waste and recovering bioenergy. The degradation of organic matter to methane consists of four basic steps: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. Correspondingly, this process requires at least four physiologically different microbial groups: hydrolyzing bacteria, fermenting bacteria, acetogenic bacteria, and two types (i.e., acetoclastic and hydrogenotrophic) of methanogenic archaea. Effective methanogenesis relies on the metabolic interaction between these microbial groups. In addition to the hydrolysis step, the conversion of the major intermediate metabolites, such as acetate and propionate, is rate-limiting. So far, two pathways for methane formation from acetate have been reported. The first one is an acetoclastic pathway whereby acetate is utilized by acetoclastic methanogens, such as *Methanosarcina* and *Methanosaeta*. The methyl and carboxyl groups of acetate are converted to CH_4 and CO_2, respectively. The second pathway includes a two-step reaction, in which acetate is first oxidized to H_2 and CO_2 by syntrophic acetate-oxidizing bacteria (SAOB) and then, the CO_2 produced is reduced to CH_4 by hydrogenotrophic methanogens in the presence of H_2 as a reducing agent. It is usually assumed that most of the CH_4 produced (up to ~67%) is derived directly from acetate (Conrad, 1999). However, in several recent studies, it has been found that syntrophic acetate oxidation (SAO) is significant under some specific environmental conditions (Hao et al., 2011; Karakashev et al., 2006; Schnürer and Nordberg, 2008; Shigematsu et al., 2004). SAO is the main pathway for acetate degradation when the acetate concentration is low (Shigematsu et al., 2003), or if some inhibitors (particularly ammonium ions) are

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present (Schnürer and Nordberg, 2008).

Because of the symbiotic relationships, isolation of syntrophic acetate-oxidizing bacteria is challenging. So far, only six SAOB, capable of forming syntrophy with hydrogenotrophic methanogens, have been isolated, including the thermophilic strain AOR (Lee and Zinder, 1988), *Thermacetogenium phaeum* (Hattori et al., 2000), and *Thermotoga lettingae* (Balk, 2002) as well as mesophilic *Clostridium ultunense* (Schnürer et al., 1996), *Syntrophaceticus schinkii* (Westerholm et al., 2010), and *Tepidanaerobacter acetatoxydans* (Westerholm et al., 2011). The knowledge of SAOB in anaerobic digesters with complex microbial communities remains quite limited. Hence, studies of SAOB at the microbial community level are necessary to clarify the microbial mechanisms of methanogenesis. In previous studies, we analyzed the microbial community structures in a chemostat fed with acetate as the sole carbon source using 16S rRNA gene clone library analysis and real-time quantitative PCR (Shigematsu et al., 2003). The $^{13}CH_4/^{12}CH_4$ and $^{13}CO_2/^{12}CO_2$ ratios in the biogas produced were determined when the microbial community was incubated with $^{13}CH_3{}^{12}COOH$ or $^{12}CH3^{13}COOH$ (Shigematsu et al., 2004). The results suggested that SAO dominated at a low dilution rate of 0.025 d^{-1}, and that the bacteria in the chemostat had a high diversity and were affiliated to four phyla: *Firmicutes*, *Bacteroidetes*, *Chloroflexi*, and candidate division OP12 (Shigematsu et al., 2003). However, the particular species that were involved in SAO were unknown, because no clone was found to be closely related to any SAOB isolated to date.

Some homoacetogenic bacteria may reverse the direction of the acetyl-CoA pathway to allow for SAO (Hattori, 2008). Formyltetrahydrofolate synthetase (FTHFS) is one of the key enzymes for reductive acetogenesis, and its coding gene has been used as an ecological biomarker for studying SAOB (Hori et al., 2011; Muller et al., 2013). However, the information regarding SAOB revealed via this way is limited because the PCR primers for amplifying FTHFS genes were designed using the limited sequences from pure cultured species. Stable isotope probing of DNA (DNA-SIP) or RNA (RNA-SIP) avoids the limits of pure culture and FTHFS gene amplification and provides a link between phylogeny and function. In recent years, DNA-SIP has been widely used to identify the functions of uncultured microorganisms. By DNA-SIP, Rui et al. (2011) studied the SAO in the rice field soil by DNA-SIP and found that some species related to *Thermacetogenium*, and species in unclassified *Thermoanaerobacteraceae*, were responsible for SAO. However, knowledge of SAO and the responsible bacteria in methanogenic reactors remain largely unknown. The objectives of the present study were to identify, for the first time, potential SAOB in a mesophilic methanogenic chemostat fed with acetate as the sole carbon source by using DNA-SIP.

Materials and Methods

Construction and operation of the anaerobic chemostat fed with acetate as the sole carbon source. The seed sludge for the construction of the chemostat (working volume of 1.8 L) was obtained from an anaerobic digester treating distillery of an alcohol plant in Sichuan Province, China. The chemostat was operated at 37°C and fed with synthetic wastewater (TOC 8000 mg/L) containing acetate as the sole carbon source at a dilution rate of 0.1 d^{-1}. The set-up of the chemostat and components of the synthetic wastewater were the same as those described in the previous study (Shigematsu et al., 2003). The microbial community in the chemostat operated stably for more than six months was used for the following experiments.

Incubation with [$^{13}C_2$] and [$^{12}C_2$] acetate. Twenty milliliters of sludge were taken from the chemostat and transferred into a 50-mL glass bottle full of N_2. The headspaces were flushed with pure N_2 for 3 min, and the bottles were sealed with a butyl rubber stopper and an aluminum cap. Cysteine-HCl was added as reducing agent and resazurin was added as anaerobic condition indicator. In order to reduce the quantity of unlabeled acetate originally present in the sludge, the bottles were pre-incubated for 24 h at 37°C while shaking. After the pre-cultivation, 4 mM (final concentration) of sodium acetate [$^{13}C_2$] (99% in ^{13}C; Cambridge Isotope Laboratories, USA) or unlabeled sodium acetate [$^{12}C_2$] (control) were added to the bottles every day during the incubation, and the bottles were named as Ace13 (fed with $^{13}C_2$) and Ace12 (fed with $^{12}C_2$), respectively. All experiments were carried out in duplicate. Gas production was measured using an Automatic Methane Potential Test System (AMPTS II, Bioprocess Control Sweden AB, Sweden).

DNA extraction and gradient fractionation. Twenty milliliters of sludge were used for DNA extraction after 7, 15 and 28 days according to the method described previously (Shigematsu et al., 2003). The DNA of the duplicates was pooled in equal amounts and used for density gradient centrifugation. Density gradient centrifugation was performed following a previously described protocol (Lueders et al., 2003). Briefly, the centrifugation solution was prepared by mixing 1.2 mL of the gradient buffer (GB) (0.1 M Tris-HCl [pH = 8.0], 0.1 M KCl, and 1 mM EDTA) containing 2.5 μg DNA and 4.8 mL of 1.9 g mL^{-1} CsCl solution. Prior to centrifugation, the average density of mixtures was determined by using a digital refractometer (AR200, Reichert, USA) and adjusted by adding a small volume of the CsCl solution or gradient buffer, if necessary. The solution was transferred to a 6.3-mL quick-seal polyallomer tube and spun in a Ti90 fixed angle rotor (Beckman, USA) at 177,000 × *g* at 20°C for 40 h, using a Beckman Coulter Optima^{TM} L-XP ultracentrifuge (Beckman Coulter, USA). Centrifuged gradients were fractionated from bottom to top into 15 equal fractions (~400 μL) with the fraction recovery system (Beckman Coulter, USA). A precisely controlled flow rate was achieved by displacing the gradient medium with sterile water at the top of the tube using a Model Pico Plus Syringe Pump (Harvard Apparatus, USA). The buoyant density (BD) of each fraction was determined by AR200 handheld refractometer (Reichert, USA). DNA was recovered by PEG6000 precipitation with glycogen and dissolved in 30 μL sterile water.

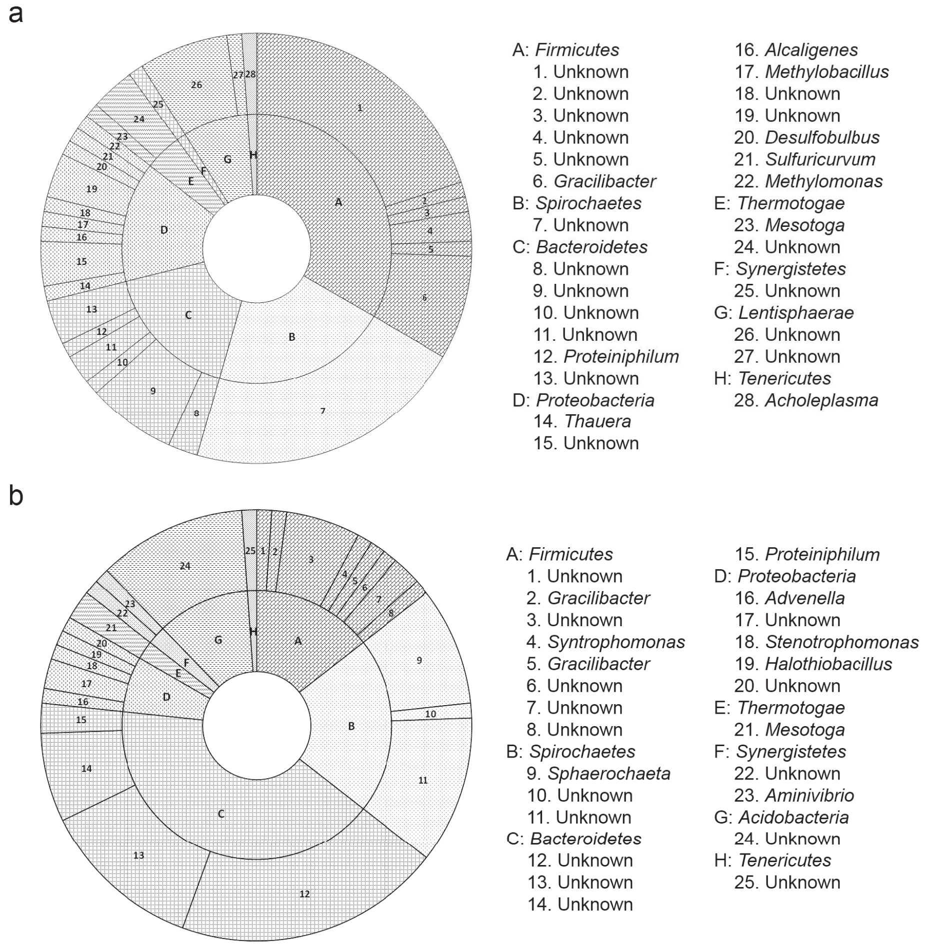

Fig. 1. Community composition of whole bacterial 16S rRNA gene clones.
(a) Original chemostat (Bac library); (b) The [$^{13}C_2$] acetate labeled system (AB library).

qPCR, cloning, sequencing, and phylogenetic analysis. In order to profile the distribution of DNA in the 15 fractions recovered, bacterial and archaeal 16S rRNA genes in all fractions were quantified by qPCR using the Eco^{TM} real-time PCR system (Illumina, USA) with primer sets Eu27f/Eu518r (Shigematsu et al., 2006b) and Arch349f/Arch806r (Takai and Horikoshi, 2000). The bacterial clone AB54 with 99.0% sequence similarity to *Proteiniphilum* sp. S2 (GenBank database accession No. KP178480) and the archaeal clone Ar26 with 100% sequence similarity to *Methanosarcina mazei* (AB973358) were used as standards for the determination of bacterial and archaeal 16S rRNA gene copies, respectively. The 20-μL reaction mixture contained 10 μL of SYBR Premix Ex Taq II (Takara, Japan), 0.8 μL of each primer solution, and 2 μL of the DNA solution.

Four bacterial clone libraries were constructed. Community DNA samples extracted from the chemostat, [$^{13}C_2$] acetate-labeled system (Ace13), and two heavy fractions (fraction 8 and 9) of Ace13, as judged by qPCR, were used for PCR amplification of bacterial 16S rRNA genes using the primer set Eu27f/1490r (Shigematsu et al., 2006b). The corresponding names of the resulting clone libraries were Bac, AB, Ace13(8), and Ace13(9). Eight archaeal clone libraries were constructed using community DNA samples from the chemostat, [$^{13}C_2$] acetate-labeled system (Ace13), and three heavy fractions 9–11 from Ace12 and Ace13, judged by qPCR, using the primer set Ar109f/Ar915r (Großkopf et al., 1998). The corresponding names of the resulting clone libraries were Arc, AA, A12-9, A12-10, A12-11, A13-9, A13-10, and A13-11. One formyltetrahydrofolate synthetase (FTHFS) gene clone library (F library) was constructed using the chemostat community DNA. FTHFS genes were amplified as described by Westerholm et al. (2011). The degenerated primer sets FTHFSf (5′-TTYACWGGHGAYTTCCATGC-3′) and FTHFSr (5′-GTATTGDGTYTTRGCCATACA-3′) were used to amplify an 1100-bp fragment of the FTHFS gene. PCR products were purified and ligated into pMD19-T Simple Vector (Takara) according to the manufacturer's instructions. Plasmids were transformed into *Escherichia coli* DH5α competent cells (Takara), and randomly selected clones were sequenced by Sangon Biotech (Shanghai, China) Co., Ltd. using ABI 3730XL DNA analyzer (Applied Biosys-tems, Foster City, CA, USA).

The sequences were analyzed by the DNAStar software package. Sequences with similarity ≥97% were classified as one operational taxonomic unit (OTU). OTU was clustered using mothur software (http://www.mothur.org/wiki/Main_Page). Phylogenetic analysis was performed using the MEGA4 software package (http://megasoftware.net/mega4/) after multiple alignments of sequences by Clustalx1.8 (http://www.clustal.org/). The phylogenetic trees were constructed using neighbor-joining methods. The confidence level for nodes was ascertained by performing a bootstrap analysis (1,000 replications). The Shannon-Wiener diversity index (H) was used as a measure of evenness and richness of the bacterial community, and was calculated from $H = \sum P_i \ln P_i$, where P_i is the proportion of genera n_i in the total bacterial community n.

Analytical methods. Parameters of suspended solids (SS), volatile suspended solids (VSS), total organic carbon (TOC), and volatile fatty acids (VFAs), were measured as described previously (Shigematsu et al., 2003).

Nucleotide sequence accession numbers. The nucleotide sequences data of the 16S rRNA gene and FTHFS gene obtained in this study are available in the GenBank database under the accession numbers of KU667175-KU667248 and KX650503-KX650506.

Results

Performance of the chemostat fed with acetate as the sole carbon source

The mesophilic chemostat fed with the synthetic wastewater with acetate as the sole carbon source was operated stably for more than six months (Supplementary Material, Fig. S1). Table S1 summarizes the average values of parameters between days 395 and 518, when the reactor operated at a dilution rate of 0.1 d^{-1}. Gas production was stable and the accumulation of acetate was negligible during this period. Acetate fed into the chemostat was completely mineralized by the microbial community in the chemostat. Sludge samples on the day 518 were used for clone library analysis and DNA-SIP experiment.

Microbial community in the chemostat operated at 0.1 d^{-1}

A bacterial and an archaeal 16S rRNA gene clone library, Bac and Arc libraries, were constructed to investigate the microbial community in the chemostat. In Bac, a total of 90 positive clones were analyzed and 28 OTUs were obtained (Fig. 1a). They were assigned to eight phyla (Table 1). Seven OTUs (30 clones, 33.33% of the total clones) were classified to the phylum *Firmicutes* (Fig. 2). Among these OTUs, five of them were affiliated to the order *Clostridiales* (11 clones, 12.22%), two OTUs to the order *Limnochordales* (19 clones, 21.11%). *Spirochaetes* was the second predominant phylum in the Bac library, represented by one OTU, Bac9 (19 clones, 21.11%) (Fig. 3). *Bacteroidetes* was the third predominant phylum in the Bac library represented by six OTUs (15 clones, 16.67%) (Fig. 3). *Proteobacteria* were the fourth predominant phylum in the Bac library, to which 9 OTUs (13 clones, 14.44%) belonged (Fig. 4). The OTUs were widely distributed in *Proteobacteria* and affiliated to four classes. All 27 positive archaeal clones from the Arc library shared 99–100% sequence similarities with *Methanosarcina mazei*. Species of *Methanosarcina mazei* can use both CO_2/H_2 and acetate, but different strains have different preferences for acetate or CO_2/H_2 (Liu et al., 1985; Mah, 1980). *Methanosarcina mazei* might mainly use acetate to produce methane in the chemostat, because no other acetoclastic methanogens were detected.

Table 1. Comparison of bacterial clone libraries of Bac, AB, Ace13(8) and Ace13(9).

Phylum	Bacterial clone library (%)			
	Bac	AB	Ace13(8)	Ace13(9)
Firmicutes	33.33	14.44		17.24
Spirochaetes	21.11	21.11		20.69
Bacteroidetes	16.67	41.11		3.45
Proteobacteria	14.44	6.67	20.00	13.79
Thermotogae	4.44	2.22		
Synergistetes	1.11	2.22		
Lentisphaerae	7.78			
Tenericutes	1.11	1.11		
Acidobacteria		11.11	80.00	34.48
Deferribacteres				3.45
Actinobacteria				6.90

A formyltetrahydrofolate synthetase gene library (F library) was constructed to investigate SAOB in the chemostat. A total of 55 positive clones were analyzed and only four OTUs were obtained (Fig. S2). The predominant OTU F203 shared a 85.0% amino acid similarity with clone 2D formyltetrahydrofolate synthetase (AEE64911) obtained from the gut of *Microcerotermes* sp. Cost008. Subdominant OTU F29 shared a 80% amino acid similarity with formyltetrahydrofolate ligase (WP_046066042) of a homoacetogenic bacterium *Clostridium scatologenes*. OTU F54 shared a 76% amino acid similarity with formyltetrahydrofolate ligase (WP_027398356) of *Anaerovorax odorimutans*. OTU F95 shared only a 54% amino acid similarity with *Thermoanaerobacterium saccharolyticum* formyltetrahydrofolate ligase (WP_045408782). All OTUs shared low amino acid similarities with FTHFSs of known SAOB, suggesting that some of these OTUs might represent novel SAOB. The very limited number of OTUs in the F library might be due to the limited universality of the primers, which were designed based on limited FTHFS gene sequences.

As described above, although the chemostat was fed with acetate as the sole carbon source, the bacterial diversity was relatively high (H = 2.76), compared with the other reactor fed with the same carbon source (H = 1.1) (Kovacik et al., 2010). With the exception of some detected fermentative bacteria responsible for sugar and protein degradation, the majority of OTUs showed a low sequence similarity to sequences of pure culture species. In addition, sequences of some OTUs showed a high similarity to sequences of uncultured clones from various environments, including methane generation reactors. No OTUs were found closely related to any pure-cultured SAOB isolated to date. The FTHFS gene sequences in the chemostat showed low values of amino acid similarity to the FTHFS gene sequences of pure-cultured SAOB. These results suggested the exciting possibility of the existence of novel acetate-oxidizing bacteria in the chemostat.

DNA-SIP

During the incubation, over 98% of acetate added was

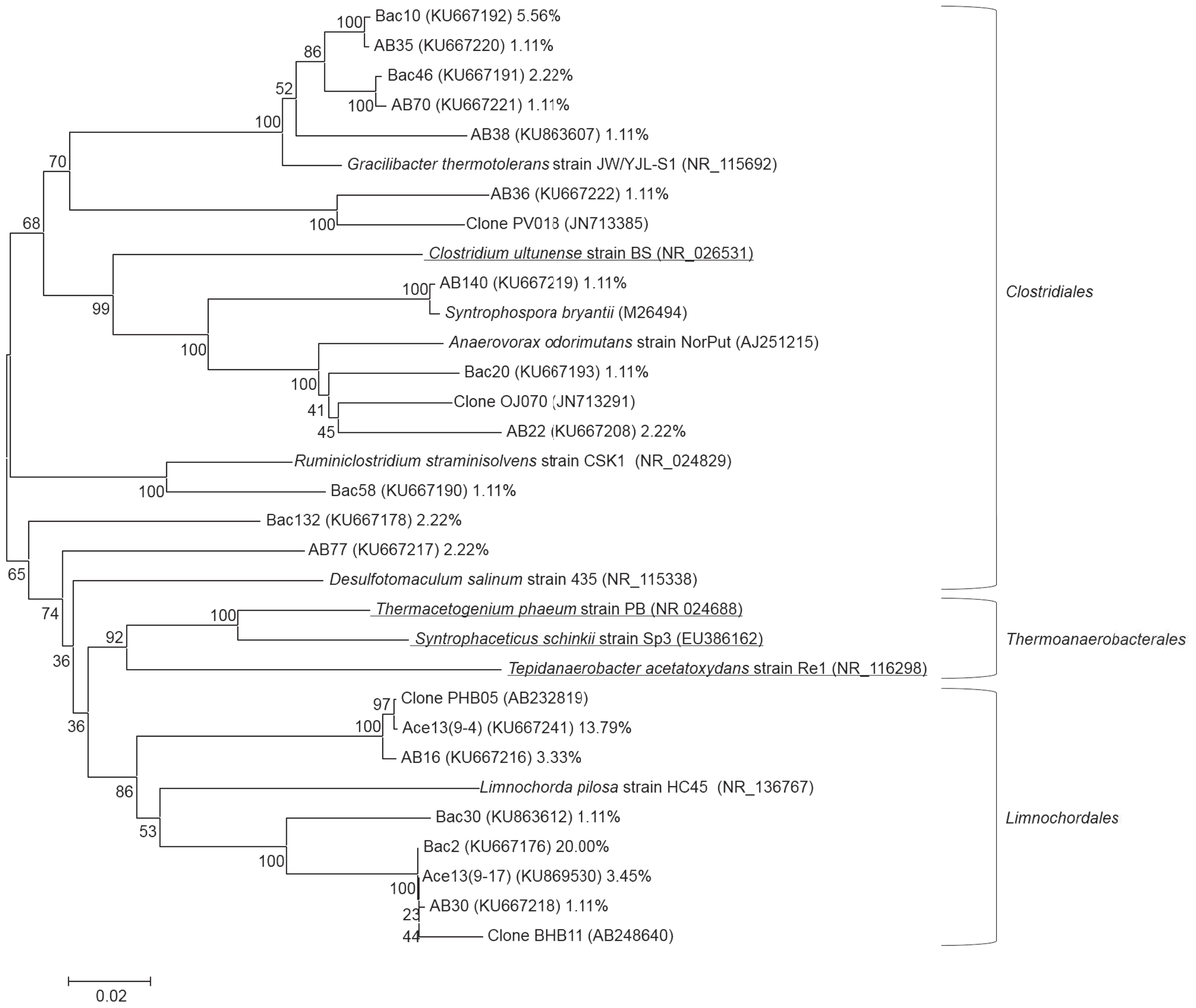

Fig. 2. Phylogenetic tree showing the genetic relationships among clones affiliated with the phylum *Firmicutes*.

The DDBJ/EMBL/GeneBank accession numbers for reference strains and clones obtained in this study are shown in parentheses in this and other figures. The percentage of clones of each OTU in corresponding library is shown after the accession number in this and other figures. The tree was constructed by the neighbor-joining method using partial sequences of 16S rRNA gene. The bar represents five substitutions of per 100 nucleotide positions.

consumed in both Ace13 and Ace12 bottles. The observed biogas production was approximately 80% of the theoretical estimates in all bottles.

Gradient centrifugation of DNA was performed for Ace12 and Ace13 DNA extracts. The quantitative distribution of bacterial and archaeal 16S rRNA genes in 15 gradients was analyzed by qPCR. After 7-d incubation, no enriched DNA was found in heavy fractions of Ace13 sample. After 15-d incubation, a small amount of DNA was detected in heavy fractions of the Ace13 sample, which means a small part of the microorganisms were labeled by [$^{13}C_2$] acetate. After 28-d incubation, DNA was enriched significantly in heavy fractions of the Ace13 sample, which suggested that cross-feeding might occur. Therefore, samples of 15-d incubation were used for further microbial community analysis. After the 15-d incubation with [$^{13}C_2$] acetate, bacterial DNA from Ace13 already showed detectable labeling in the heavy fraction (1.73–1.76 g·mL^{-1}) (Fig. 5a), whereas the amount of bacterial DNA in the heavy fraction of Ace12 (1.73–1.76 g·mL^{-1}) was negligible (Fig. 5a). This result indicated that some bacteria assimilated [$^{13}C_2$] acetate, and their DNA molecules were successfully labeled by ^{13}C. The distribution of archaeal 16S rRNA gene copies suggested that archaea were also labeled successfully (Fig. 5b).

Comparison of microbial communities in Ace13 and Ace12

From Ace13, 90 bacterial clones (AB library) were analyzed (Fig. 1b). Forty-one percent of the total clones belonged to Bacteroidetes, followed by *Spirochaetes* (21.11%), *Firmicutes* (14.44%), *Acidobacteria* (11.11%), *Proteobacteria* (6.67), *Synergistetes* (2.22%), *Thermotogae* (2.22%) and *Tenericutes* (1.11%) (Table 1). Among the 57 archaeal clones (AA library), 89% belonged to *Methanosarcina*, and the remaining archaea were represented by *Methanoculleus* (3.51%), *Methanobrevibacter* (3.51%), *Methanococcus* (1.75%), and candidatus *Methanoplasma* (1.75%). During the labeling incubation, the carbon supply was much more limited than in the chemostat; hence, the microbial community after the 15-d incubation changed to some extent, compared to the com-

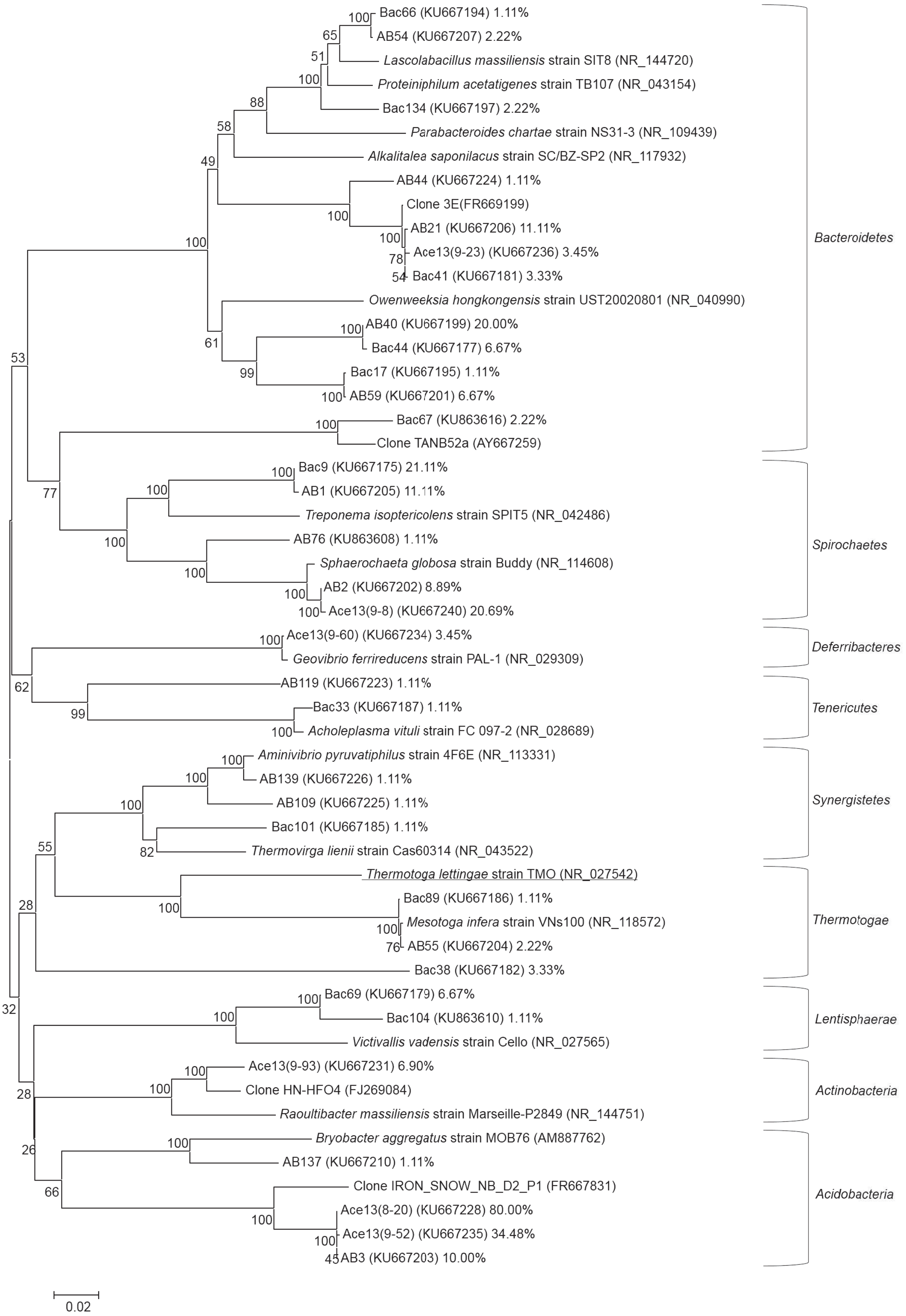

Fig. 3. Phylogenetic tree showing the genetic relationships among clones affiliated with the phylums except *Firmicutes* and *Proteobacteria*.

The tree was constructed by the neighbor-joining method using partial sequences of 16S rRNA gene. The bar represents two substitutions of per 100 nucleotide positions.

position of the Bac and Arc libraries (Table 1, Figs. 2–4).

Based on the distribution patterns of archaeal 16S rRNA gene copies, the "heavy" DNA fractions with buoyant density (BD) values of 1.71–1.74 g·mL^{-1} (fractions 9 (the heaviest), 10, and 11, Fig. 5a) were selected from Ace13 and Ace12 to construct archaeal 16S rRNA gene clone libraries. As shown in Table 2, *Methanosarcina* was exclusively dominant in all six libraries. Compared with that

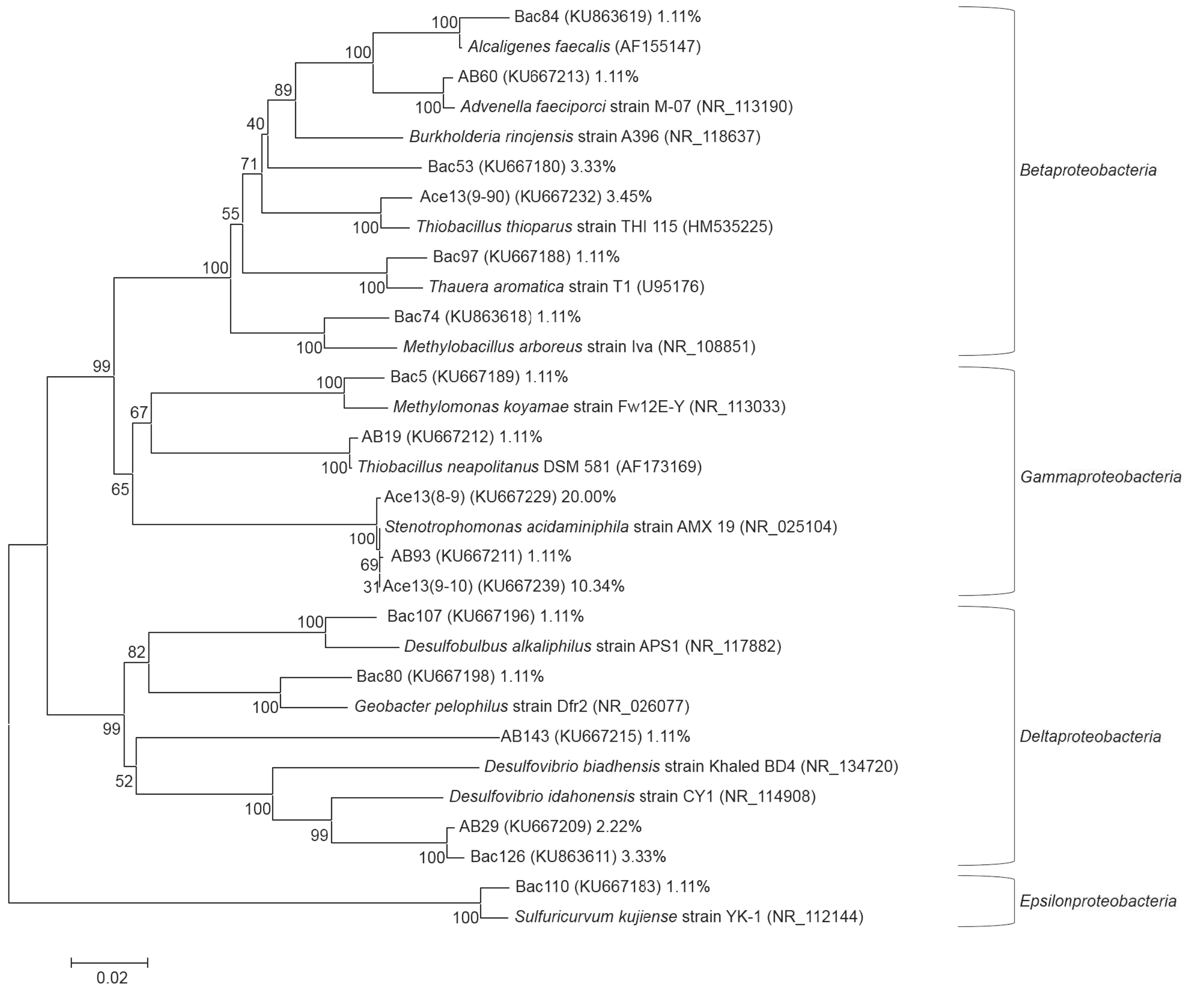

Fig. 4. Phylogenetic tree showing the genetic relationships among clones affiliated with the phylum *Proteobacteria*.

The tree was constructed by the neighbor-joining method using partial sequences of 16S rRNA gene. The bar represents two substitutions of per 100 nucleotide positions.

no clone of hydrogenotrophic methanogens was revealed in A12-9, clones of hydrogenotrophic *Methanoculleus* and unclassified *Methanococci* accounted for 12.90% and 6.45%, respectively, of the total clones in A13-9. The percentage of unclassified *Methanococci* clones in A13-10 was 16.67%, which was much higher than that in A12-10 (2.7%). In addition, clones of *Methanobrevibacter* were found in A13-10, but not in A12-10. The methanogen communities in the lower BD fractions A12-11 and A13-11 were similar. These results indicated that in addition to acetoclastic methanogens, hydrogenotrophic methanogens were labeled, suggesting that the syntrophic acetate degradation pathway existed in the chemostat. The order of the labeling of three genera of hydrogenotrophic methanogens suggested that *Methanoculleus* was the primary partner of acetate-oxidizing bacteria.

Based on the distribution patterns of bacterial 16S rRNA gene copies, the "heavy" DNA fractions with BD values of 1.73–1.75 g·mL^{-1} (fraction 8 (the heaviest) and 9 (second heaviest)), Fig. 5b) from both Ace12 and Ace13 were selected to construct bacterial 16S rRNA gene clone libraries. No PCR product was obtained when Ace12 fractions 8 or 9 were used as template, suggesting that the quantity of bacterial DNA in these two fractions was too low to be amplified by the primer set Eu27f/1490r. PCR products from Ace13 fractions 8 and 9 were obtained, and bacterial clone libraries Ace13(8) and Ace13(9) were constructed. Clones assigned to *Acidobacteria* and *Proteobacteria* were dominant in both Ace13(8) and Ace13(9) (Tables 1 and 3). In Ace13(9), clones assigned to *Spirochaetes*, *Firmicutes*, *Actinobacteria*, *Bacteroidetes* and *Deferribacteres* were also detected. The majority of the clones showed a low sequence similarity to pure-cultured strains (Table 3). These labeled bacteria might be novel acetate-utilizers (AUB) or SAOB responsible for acetate degradation in the chemostat.

The two OTUs in Ace13(8) were also found in Ace13(9). The predominant OTU, Ace13(8-20) and Ace13(9-52), which accounted for 80% and 34.48% clones of the Ace13(8) and Ace13(9), respectively (Table 3), shared only a 80% sequence similarity with *Bryobacter aggregatus* strain MOB76 (AM887762) and a 92% sequence similarity with clone IRON_SNOW_NB_D2_P1 (FR667831) from an acidic coal-mining-associated lake. One OTU, Ace13(8-9) and Ace13(9-10), shared 99% sequence similarity with the *Stenotrophomonas acidaminiphila* strain AMX 19 (NR_025104), a strictly aerobic bacterium isolated from an upflow anaerobic sludge blanket reactor

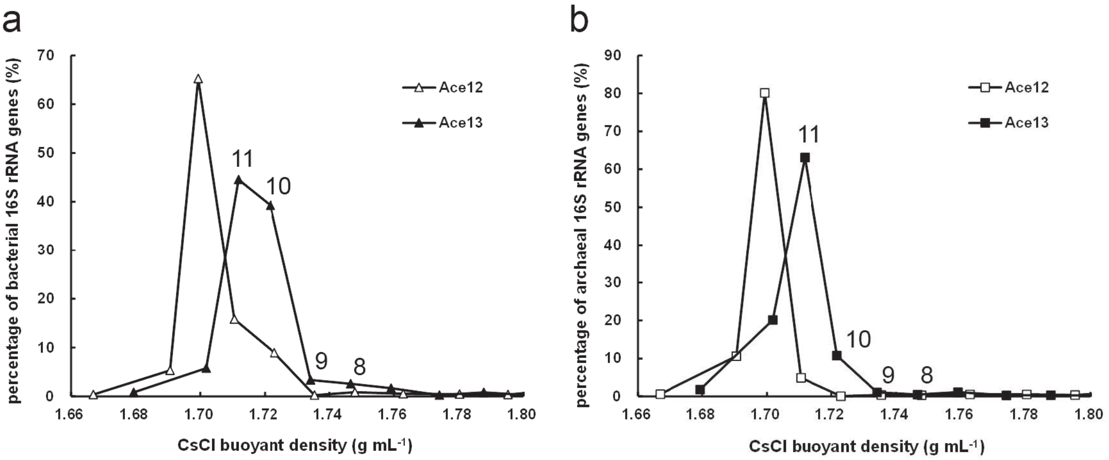

Fig. 5. Relative abundance of bacterial (a) and archaeal (b) 16S rRNA genes in the gradient fractions.

Table 2. Distribution of archaeal 16S rRNA clones retrieved from heavy fractions of DNA-SIP experiment.

Genus	Archaeal clone library					
	A12-9	A13-9	A12-10	A13-10	A12-11	A13-11
Methanosarcina	100.00%	80.65%	81.08%	75.00%	96.67%	97.44%
Methanoculleus	0	12.90%	13.51%	5.56%	0	0
unclassified *Methanococci*	0	6.45%	2.70%	16.67%	3.33%	2.56%
Methanobrevibacter	0	0	0	2.78%	0	0
Methanomassiliicoccus	0	0	2.70%	0	0	0

(Assih et al., 2002). This OTU accounted for 20.00% and 10.34% of the total clones in Ace13(8) and Ace13(9), respectively.

In addition to OTUs common with those in Ace13(8), seven unique OTUs were identified in Ace13(9) (Table 3). Two OTUs (Ace13(9-17), Ace13(9-4)) were classified in *Firmicutes*, and both of them assigned to the order *Limnochordales*. However, the two OTUs had low sequence similarities with those pure-cultured SAOB. Among six SAOB strains isolated so far, five of them were *Firmicutes* species. OTU Ace13(9-4), which accounted for 13.79% of the Ace13(9) clones, shared a 99% sequence similarity with the uncultured clone PHB05 (AB232819) obtained from a mesophilic propionate-degrading methanogenic reactor (Shigematsu et al., 2006a) (Table 3, Fig. 2). OTU Ace13(9-17) shared a 97% sequence similarity with the uncultured clone BHB11 (AB248640) obtained from a mesophilic butyrate-degrading methanogenic reactor (Tang et al., 2007). The same OTU were detected in Bac and accounted for 20.00% of the clones in Bac (Table 3, Fig. 2).

OTU Ace13(9-23) accounted for 3.45% of the clones in Ace13(9). It shared a low sequence similarity of 85% with *Parabacteroides chartae* strain NS31-3 (NR_109439) but a high sequence similarity of 99% with the uncultured clone 3E (FR669199) obtained from the biocathode of a hydrogen-producing microbial electrolysis cell (Croese et al., 2011). The same OTU was also found in Bac, where it accounted for 3.33% of the total clones (Table 3, Fig. 3). OTU Ace13(9-90) was classified in *Proteobacteria*, and it shared a 98% sequence similarity with *Thiobacillus thioparus* strain THI 115 (HM535225) (Table 3, Fig. 4). OTU Ace13(9-8), which accounted for 20.69% of the clones in the Ace13(9) library, was affiliated to *Spirochaetes* and shared a 99% sequence similarity with *Sphaerochaeta globosa* strain Buddy (NR_114608) (Table 3, Fig. 3). OTU Ace13(9-93) was affiliated to *Actinobacteria* and shared a 91% sequence similarity with *Raoultibacter massiliensis* strain Marseille-P2849 (NR_144751). This OTU was closely related (96% sequence similarity) to the uncultured clone HN-HFO4 (FJ269084) obtained from enrichment cultures of dissimilatory iron-reducing bacteria (Wang et al., 2009). OTU Ace13(9-60) was affiliated to *Deferribacteres* and shared a 99% sequence similarity with *Geovibrio ferrireducens* strain PAL-1 (NR_029309), which was isolated from the surface sediment of a hydrocarbon-contaminated ditch (Caccavo et al., 1996).

Discussion

Acetate is one of the major intermediates of anaerobic degradation under methanogenic conditions. The research on the degradation of acetate has been mostly focused on methanogens and known SAOB. However, the knowledge regarding SAOB is limited, because they cannot be easily obtained in pure cultures due to the difficulty of their isolation. SIP provides the means to study the relationship between environmental functions and specific microbial community members.

In this study, a mesophilic anaerobic chemostat fed with acetate as the sole carbon source was constructed, and the

Table 3. Distribution of bacterial 16S rRNA clones retrieved from "most heavy" and "second heavy" fraction of DNA-SIP experiment.

	Clone	Closest relatives	Sequence similarity (%)	Relative abundance (%)	Relative abundance in Bac library (%)
Ace13(8)	*Proteobacteria*				
	Ace13(8-9)	*Stenotrophomonas acidaminiphila* strain AMX19 (NR_025104)	99	20.00	
	Acidobacteria				
	Ace13(8-20)	*Bryobacter aggregatus* strain MOB76 (AM887762)	80	80.00	
		uncultured bacterium clone IRON_SNOW_NB_D2_P1 (FR667831)	92		
Ace13(9)	*Proteobacteria*				
	Ace13(9-10)	*Stenotrophomonas acidaminiphila* strain AMX 19 (NR_025104)	100	10.34	
	Ace13(9-90)	*Thiobacillus thioparus* strain THI 115 (HM535225)	98	3.45	
	Acidobacteria				
	Ace13(9-52)	*Bryobacter aggregatus* strain MOB76 (AM887762)	80	34.48	
		uncultured bacterium clone IRON_SNOW_NB_D2_P1 (FR667831)	92		
	Firmicutes				
	Ace13(9-17)	*Limnochorda pilosa* strain HC45 (NR_136767)	86	3.45	20.00
		uncultured bacterium clone BHB11 (AB248640)	97		
	Ace13(9-4)	*Limnochorda pilosa* strain HC45 (NR_136767)	85	13.79	
		uncultured bacterium clone PHB05 (AB232819)	99		
	Bacteroidetes				
	Ace13(9-23)	*Parabacteroides chartae* strain NS31-3 (NR_109439)	85	3.45	3.33
		uncultured bacterium clone 3E (FR669199)	99		
	Spirochaetes				
	Ace13(9-8)	*Sphaerochaeta globosa* strain Buddy (NR_114608)	99	20.69	
	Actinobacteria				
	Ace13(9-93)	*Raoultibacter massiliensis* strain Marseille-P2849 (NR_144751)	91	6.90	
		uncultured bacterium clone HN-HFO4 (FJ269084)	96		
	Deferribacteres				
	Ace13(9-60)	*Geovibrio ferrireducens* strain PAL-1 (NR_029309)	99	3.45	

emerged microbial community was analyzed. The results suggested that the community was complex and had a high diversity (H = 2.76). Bacteria that did not take part in acetate metabolism but played roles in the degradation of components of dead cells might comprise a considerable proportion of the community (Narihiro et al., 2015). FTHFS gene analysis suggested that the chemostat probably contained novel acetate-oxidizing bacterial species. Therefore, DNA-SIP was applied to identifying the bacteria that assimilated acetate in the chemostat. Due to a long period of domestication of the microbial community in the chemostat with acetate, the labeling time in the present study was much shorter than that in studies targeting other environmental microbial communities, e.g., the community of the rice field soil (Liu and Conrad, 2010). After 15 days of incubation, labeled DNA was obtained at a density higher than 1.74 g mL^{-1}.

OTUs in Ace13(8) and Ace13(9) were diverse but shared low sequence similarities with known SAOB, indicating the possibility of the existence of novel SAOB in the chemostat. Two OTUs, Ace13(9-17) and Ace13(9-23), detected in heavy fractions were found in Bac. These Two OTUs accounted for 23.33% of the total clones in the Bac library (Figs. 2–4). This observation suggested a high ratio of AUB in the chemostat. The two OTUs detected in both the Bac library and the Ace13(9) library might be the major AUB in the chemostat. OTU Bac2, which accounted for 20% of the total clones in Bac, was the same as the OTU Ace13(9-17) (Fig. 2). This OTU had a 97% sequence similarity with the clone BHB11 (AB248640) obtained from a mesophilic butyrate-fed anaerobic bioreactor (Tang et al., 2007). Under methanogenic conditions, butyrate is degraded to acetate and H_2, which creates appropriate conditions for SAOB. Thus, taking into account the observations in the present study, bacterium represented by clone BHB11 (AB248640) most likely oxidized acetate in the mesophilic butyrate-fed anaerobic bioreactor. OTU Bac2 was probably the major SAOB in the chemostat. OTU Bac41, which accounted for 3.33% of the clones in Bac, was the same as the OTU Ace13 (9-23). It shared a high sequence similarity of 99% with clone 3E (FR669199) obtained from the biocathode of a hydrogen-producing microbial electrolysis cell (MEC) fed with acetate (Croese et al., 2011) (Table 3) (Fig. 3). The inoculum of the MEC, from which clone 3E was obtained, represented a mixed microbial community fed with a mixture of acetate and hydrogen from its early stage (Croese et al., 2011). Therefore, considering the results of the present study, it could be deduced that bacterium represented by clone 3E probably oxidized acetate to produce hydrogen in the MEC, and OTU Bac41 probably syntrophically oxidized acetate with hydrogenotrophic methanogens in the chemostat of the present study. However, further study in needed to confirm whether OTU Bac2 and OTU Bac41 are SAOBs.

Eight OTUs that were labeled by [$^{13}C_2$] acetate were not detected in Bac (Table 3) (Figs. 2–4). This result may be explained by the low abundance of these OTUs in the chemostat and limitations of the clone library analysis. In the phylum *Firmicutes*, OTU Ace13(9-4) showed a 99% sequence similarity with the uncultured clone PHB05 (AB232819). Clone PHB05 was obtained from a mesophilic propionate-degrading methanogenic reactor

(Shigematsu et al., 2006a), in which acetate was the product of propionate degradation. Considering those findings and the results of the present study, it is suggested that OTU Ace13(9-4) and clone PHB05 may be possible SAOB, which need further investigation. OTU Ace13(9-90) shared a 98% sequence similarity with the sulfur-oxidizing bacteria *Thiobacillus thioparus* strain THI 115 (HM535225), which might utilize acetate or CO_2 in the reactor. In the class *Gammaproteobacteria*, OTU Ace13(8-9) and OTU Ace13(9-10) clustered together and shared a 99–100% sequence similarity with aerobic bacterium *Stenotrophomonas acidaminiphila* strain AMX 19 (NR_025104) (Assih et al., 2002). However, its growth was possible also under anoxic conditions, when it was coupled to nitrate and nitrite consumption, with acetate as the sole carbon source (Assih et al., 2002). In the phylum *Spirochaetes*, OTU Ace13(9-8) shared a 99% sequence similarity with *Sphaerochaeta globosa* strain Buddy (NR_114608) (Fig. 3). Ito et al. (2011) reported that bacteria belonging to *Synergistes* group 4 can utilize acetate with considerable efficiency. Thus, the *Sphaerochaeta*-related bacterium detected in the present study may have a function similar to that of the *Synergistes* group 4 bacteria. In the phylum *Deferribacteres*, OTU Ace13(9-60) shared a 99% sequence similarity with *Geovibrio ferrireducens* strain PAL-1 (NR_029309) isolated from the surface sediment of a hydrocarbon-contaminated ditch (Caccavo et al., 1996). This bacterium is an obligately anaerobic, non-fermentative vibrio that can grow in an artificial medium with acetate as electron donor, whereas ferric pyrophosphate, ferric oxyhydroxide, ferric citrate, Co(III)-EDTA, or elemental sulfur can serve as the sole electron acceptor (Caccavo et al., 1996). In the phylum *Actinobacteria*, OTU Ace13(9-93) shared a 96% sequence similarity with the uncultured clone HN-HFO4 (FJ269084) obtained from enrichment culture of dissimilatory iron-reducing bacteria (Wang et al., 2009), which were fed with acetate as the sole carbon source (Wang et al., 2009). OTU Ace13(8-20) and OTU Ace13(9-52) clustered together and shared only a 92% sequence similarity with the most closely related clone IRON_SNOW_NB_D2_P1 (FR667831) obtained from an acidic coal mining-associated lake (Reiche et al., 2011). All these labeled OTUs were related to acetate utilization but they accounted only for a small percentage of bacteria in the chemostat.

In this study, the diversity of labeled bacteria was higher than expected, which is consistent with the results of other studies. Hao et al. (2015) investigated the influence of ammonia on microbial communities in thermophilic methanogenic reactors by DNA-SIP and observed that representatives of seven classes were labeled by ^{13}C-acetate, including *Clostridia*, *Synergistia*, *Thermotogae*, *Spirochaetes*, *Mollicutes*, *Chlorobia*, and *Betaproteobacteria* species. Na et al. (2015) investigated the microbes incorporating ^{13}C-labeled acetate in a sulfate-reducing sediment and observed that 12 OTUs were labeled by ^{13}C-acetate, including unclassified *Desulfobacteraceae*, unclassified *Clostridiaceae*, *Desulfovibrio*, *Desulfuromusa* as well as species from other families. A number of bacteria were labeled by ^{13}C-acetate in different environments, which indicates a high diversity of AOB. However, it is, to some extent, difficult to rule out cross-feeding and activity of some fermentative microbes that feed on decaying cells that had assimilated ^{13}C-acetate. In the present study, due to the long-term acclimation of the microbial community in the chemostat with acetate, the label time of 15 days was much shorter than that in the environment samples, which may have reduced to some extent the probability of cross-feeding. In fact, a 7-day label time did not result in heavy DNA. It was deducible, because the chemostat operated at a dilution rate of 0.1 d^{-1} and the specific growth rate (μ value) of the microorganisms in the reactor should be around 0.1 d^{-1}, corresponding to a generation time of 6.9 d. Hence, a label period longer than 7 days is necessary for the generation of heavy DNA. However, further studies are required to confirm the acetate oxidation function of the labeled bacteria in the present study.

With regards to the Archaea, the hydrogenotrophic methanogens *Methanoculleus*, unclassified *Methanococci*, and *Methanobrevibacter* were concentrated in the heavy fractions of ^{13}C-samples (fractions 9, 10) (Table 3), which was another proof of the presence of SAO and hydrogenotrophic methanogenesis in the chemostat. A previous study demonstrated involvement of *Methanoculleus* in the process of syntrophic oxidation by acetate-oxidizing syntrophs and hydrogenotrophic methanogens in the primary pathway of total methanogenesis (Shigematsu et al., 2004). *Methanoculleus* may have a high hydrogen affinity, because the concentration of hydrogen must be very low for the oxidation of acetate to be thermodynamically feasible (Schnürer et al., 1999). The acetate-oxidizing ability of cultured acetate-oxidizing syntrophs *Syntrophaceticus schinkii* (Westerholm et al., 2010) and *Tepidanaerobacter acetatoxydans* (Westerholm et al., 2011) was observed during their co-cultivation with *Methanoculleus* sp. Detection of the presence of hydrogenotrophic methanogens in the heavy fractions in the present study supported the notion that some of ^{13}C-labeled bacteria participated in syntrophic acetate oxidation in the chemostat.

In conclusion, acetoclastic methanogenesis and acetate oxidization/hydrogenotrophic methanogenesis coexisted in the mesophilic chemostat fed with acetate, operated at a dilution rate of 0.1 d^{-1}. OTU Ace13(9-17) (KU869530), Ace13(9-4) (KU667241) and Ace13(9-23) (KU667236), which assigned to the phylum *Firmicutes* and *Bacteroidetes* were probably syntrophic acetate-oxidizing bacteria in the chemostat. Species in the phyla *Proteobacteria*, *Deferribacteres*, *Acidobacteria*, *Spirochaetes* and *Actinobacteria* were probably capable of utilizing acetate for their growth. *Methanoculleus* was likely to be the preferred hydrogenotrophic methanogen for syntrophy with acetate-oxidizing bacteria in the chemostat.

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Supplementary Materials

Supplementary figures and table are available in our J-STAGE site (http://www.jstage.jst.go.jp/browse/jgam).

References

Assih, E. A., Ouattara, A. S., Thierry, S., Cayol, J. L., Labat, M. et al. (2002) *Stenotrophomonas acidaminiphila* sp nov., a strictly aerobic bacterium isolated from an upflow anaerobic sludge blanket (UASB) reactor. *Int. J. Syst. Evol. Microbiol.*, **52**, 559–568.

Balk, M. (2002) *Thermotoga lettingae* sp. nov., a novel thermophilic, methanol-degrading bacterium isolated from a thermophilic anaerobic reactor. *Int. J. Syst. Evol. Microbiol.*, **52**, 1361–1368.

Caccavo, F., Jr., Coates, J. D., Rossello-Mora, R. A., Ludwig, W., Schleifer, K. H. et al. (1996) *Geovibrio ferrireducens*, a phylogenetically distinct dissimilatory Fe(III)-reducing bacterium. *Arch. Microbiol.*, **165**, 370–376.

Conrad, R. (1999) Contribution of hydrogen to methane production and control of hydrogen concentrations in methanogenic soils and sediments. *FEMS Microbiol. Ecol.*, **28**, 193–202.

Croese, E., Pereira, M. A., Euverink, G.-J. W., Stams, A. J., and Geelhoed, J. S. (2011) Analysis of the microbial community of the biocathode of a hydrogen-producing microbial electrolysis cell. *Appl. Microbiol. Biotechnol.*, **92**, 1083–1093.

Großkopf, R., Janssen, P. H., and Liesack, W. (1998) Diversity and structure of the methanogenic community in anoxic rice paddy soil microcosms as examined by cultivation and direct 16S rRNA gene sequence retrieval. *Appl. Environ. Microbiol.*, **64**, 960–969.

Hao, L. P., Lu, F., He, P. J., Li, L., and Shao, L. M. (2011) Predominant contribution of syntrophic acetate oxidation to thermophilic methane formation at high acetate concentrations. *Environ. Sci. Technol.*, **45**, 508–513.

Hao, L., Lü, F., Mazéas, L., Desmond-Le Quéméner, E., Madigou, C. et al. (2015) Stable isotope probing of acetate fed anaerobic batch incubations shows a partial resistance of acetoclastic methanogenesis catalyzed by *Methanosarcina* to sudden increase of ammonia level. *Water Res.*, **69**, 90–99.

Hattori, S. (2008) Syntrophic acetate-oxidizing microbes in methanogenic environments. *Microbes Environ.*, **23**, 118–127.

Hattori, S., Kamagata, Y., Hanada, S., and Shoun, H. (2000) *Thermacetogenium phaeum* gen. nov., sp. nov., a strictly anaerobic, thermophilic, syntrophic acetate-oxidizing bacterium. *Int. J. Syst. Evol. Microbiol.*, **50**, 1601–1609.

Hori, T., Sasaki, D., Haruta, S., Shigematsu, T., Ueno, Y. et al. (2011) Detection of active, potentially acetate-oxidizing syntrophs in an anaerobic digester by flux measurement and formyltetrahydrofolate synthetase (FTHFS) expression profiling. *Microbiology*, **157**, 1980–1989.

Ito, T., Yoshiguchi, K., Ariesyady, H. D., and Okabe, S. (2011) Identification of a novel acetate-utilizing bacterium belonging to *Synergistes* group 4 in anaerobic digester sludge. *ISME J.*, **5**, 1844–1856.

Karakashev, D., Batstone, D. J., Trably, E., and Angelidaki, I. (2006) Acetate oxidation is the dominant methanogenic pathway from acetate in the absence of *Methanosaetaceae*. *Appl. Environ. Microbiol.*, **72**, 5138–5141.

Kovacik, W. P., Scholten, J. C. M., Culley, D., Hickey, R., Zhang, W. et al. (2010) Microbial dynamics in upflow anaerobic sludge blanket (UASB) bioreactor granules in response to short-term changes in substrate feed. *Microbiology*, **156**, 2418–2427.

Lee, M. J. and Zinder, S. H. (1988) Isolation and characterization of a thermophilic bacterium which oxidizes acetate in syntrophic association with a methanogen and which grows acetogenically on H(2)-CO(2). *Appl. Environ. Microbiol.*, **54**, 124–129.

Liu, F. and Conrad, R. (2010) *Thermoanaerobacteriaceae* oxidize acetate in methanogenic rice field soil at 50°C. *Environ. Microbiol.*, **12**, 2341–2354.

Liu, Y., Boone, D. R., Sleat, R., and Mah, R. A. (1985) *Methanosarcina mazei* LYC, a new methanogenic isolate which produces a disaggregating enzyme. *Appl. Environ. Microbiol.*, **49**, 608–613.

Lueders, T., Manefield, M., and Friedrich, M. W. (2003) Enhanced sensitivity of DNA- and rRNA-based stable isotope probing by fractionation and quantitative analysis of isopycnic centrifugation gradients. *Environ. Microbiol.*, **6**, 73–78.

Mah, R. A. (1980) Isolation and characterization of *Methanococcus mazei*. *Current Microbiology*, **3**, 321–326.

Muller, B., Sun, L., and Schnürer, A. (2013) First insights into the syntrophic acetate-oxidizing bacteria—a genetic study. *Microbiologyopen*, **2**, 35–53.

Na, H., Lever, M. A., Kjeldsen, K. U., Schulz, F., and Jørgensen, B. B. (2015) Uncultured *Desulfobacteraceae* and *Crenarchaeotal* group C3 incorporate ^{13}C-acetate in coastal marine sediment. *Environ. Microbiol. Rep.*, **7**, 614–622.

Narihiro, T., Nobu, M. K., Kim, N. K., Kamagata, Y., and Liu, W. T. (2015) The nexus of syntrophy-associated microbiota in anaerobic digestion revealed by long-term enrichment and community survey. *Environ. Microbiol.*, **17**, 1707–1720.

Reiche, M., Lu, S., Ciobotă, V., Neu, T. R., Nietzsche, S. et al. (2011) Pelagic boundary conditions affect the biological formation of iron-rich particles (iron snow) and their microbial communities. *Limnol. Oceanogr.*, **56**, 1386–1398.

Rui, J., Qiu, Q., and Lu, Y. (2011) Syntrophic acetate oxidation under thermophilic methanogenic condition in Chinese paddy field soil. *FEMS Microbiol. Ecol.*, **77**, 264–273.

Schnürer, A. and Nordberg, A. (2008) Ammonia, a selective agent for methane production by syntrophic acetate oxidation at mesophilic temperature. *Water Sci. Technol.*, **57**, 735–740.

Schnürer, A., Schink, B., and Svensson, B. H. (1996) *Clostridium ultunense* sp. nov., a mesophilic bacterium oxidizing acetate in syntrophic association with a hydrogenotrophic methanogenic bacterium. *Int. J. Syst. Evol. Microbiol.*, **46**, 1145–1152.

Schnürer, A., Zellner, G., and Svensson, B. H. (1999) Mesophilic syntrophic acetate oxidation during methane formation in biogas reactors. *FEMS Microbiol. Ecol.*, **29**, 249–261.

Shigematsu, T., Tang, Y., Kawaguchi, H., Ninomiya, K., Kijima, J. et al. (2003) Effect of dilution rate on structure of a mesophilic acetate-degrading methanogenic community during continuous cultivation. *J. Biosci. Bioeng.*, **96**, 547–558.

Shigematsu, T., Tang, Y., Kobayashi, T., Kawaguchi, H., Morimura, S. et al. (2004) Effect of dilution rate on metabolic pathway shift between aceticlastic and nonaceticlastic methanogenesis in chemostat cultivation. *Appl. Environ. Microbiol.*, **70**, 4048–4052.

Shigematsu, T., Era, S., Mizuno, Y., Ninomiya, K., Kamegawa, Y. et al. (2006a) Microbial community of a mesophilic propionate-degrading methanogenic consortium in chemostat cultivation analyzed based on 16S rRNA and acetate kinase genes. *Appl. Microbiol. Biotechnol.*, **72**, 401–415.

Shigematsu, T., Tang, Y., Mizuno, Y., Kawaguchi, H., Morimura, S. et al. (2006b) Microbial diversity of mesophilic methanogenic consortium that can degrade long-chain fatty acids in chemostat cultivation. *J. Biosci. Bioeng.*, **102**, 535–544.

Takai, K. and Horikoshi, K. (2000) Rapid detection and quantification of members of the archaeal community by quantitative PCR using fluorogenic probes. *Appl. Environ. Microbiol.*, **66**, 5066–5072.

Tang, Y., Shigematsu, T., Morimura, S., and Kida, K. (2007) Effect of dilution rate on the microbial structure of a mesophilic butyrate-degrading methanogenic community during continuous cultivation. *Appl. Microbiol. Biotechnol.*, **75**, 451–465.

Wang, X. J., Yang, J., Chen, X. P., Sun, G. X., and Zhu, Y. G. (2009) Phylogenetic diversity of dissimilatory ferric iron reducers in paddy soil of Hunan, South China. *J. Soils Sediments*, **9**, 568–577.

Westerholm, M., Roos, S., and Schnürer, A. (2010) *Syntrophaceticus schinkii* gen. nov., sp. nov., an anaerobic, syntrophic acetate-oxidizing bacterium isolated from a mesophilic anaerobic filter. *FEMS Microbiol. Lett.*, **309**, 100–104.

Westerholm, M., Roos, S., and Schnürer, A. (2011) *Tepidanaerobacter acetatoxydans* sp. nov., an anaerobic, syntrophic acetate-oxidizing bacterium isolated from two ammonium-enriched mesophilic methanogenic processes. *Syst. Appl. Microbiol.*, **34**, 260–266.